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Studies have shown that apoptosis and survival pathways in response to DNA damage play a critical role in breast cancer development and progression. 90% of breast cancer cases are sporadic where mutations of BRCA1/2 have not been detected. Other breast cancer genes must exist. Our group has approached the issue in two ways, to utilize a novel genetic method to screen for genes involved in DNA-damage induced apoptosis; and large-scale identification of factors that interact with BARD1. We have established and utilized a novel retrovirus-based genetic screen system to search for genes that would confer resistance to DNA dmage induced apoptosis. Multiple clones have been isolated from this genetic screen. Among the genes identified is the protein kinase Lyn which is important in DNA-damage responses. Further studies are underway to further identify other genes. To further elucidate the pathways mediated by BARD1, we employed the RNA interference assay. Furthermore, we are in the process of identifying other factors that may interact with BARD1. Several factors have emerged from this study and are being examined. The information obtained from our studies should prove useful for developing new and effective screening strategies, drug targets, and treatment for breast cancer.

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Introduction

Understanding the molecular and cellular mechanisms that trigger breast cancer is essential to the prevention and treatment of this disease. The initiation and progression of breast cancer is likely the result of dysregulation of both oncogenes and tumor suppressor genes (1, 2). Mutations of these genes can cause defects in cellular survival and proliferation, genomic integrity, and sensitivity to DNA damage. However, few genes that regulate DNA damage induced cell death are known to date, and even less is known as to how they interconnect with the apoptosis and survival pathways. We have proposed to establish a genetic system to screen for genes that regulate survival in cultured cells through high-efficiency mutagenesis using Enhanced Retroviral Mutagens (ERM) (3). Due to the random nature of retroviral integration, endogenous genes involved in cell survival signaling cascades may be activated or inactivated by ERM. The targeted gene loci are marked by retroviral integration thereby allowing quick isolation of the candidate genes. The overall objective of this proposal is to identify and study genes that allow the survival of normal and cancerous breast cells. The physiological roles of these genes and their interactions with known signaling pathways will be investigated. Genetic screens will be performed to search for survival genes in response to DNA damage. And the function and signaling mechanisms of BARD1 in breast cancer cell survival will also be examined. The proposed studies should help in our understanding of the molecular basis underlying cell survival signaling and breast cancer as well as provide new therapeutic targets for the cure of this disease.

Body

For Task 1, we have proposed to isolate mammalian genes involved in breast cancer cell survival. This will be achieved by establishing Enhanced Retrovirus Mutagen (ERM)-mediated genetic screen and analyzing isolated clones, by establishing secondary screens using human breast epithelial cells to confirm the role the cloned genes in DNA-damage-induced apoptosis, and by identifying the candidate genes targeted by ERM

In order to ientify genes that mediate DNA-damage induced apoptosis, we employed a novel retrovirus-based genetic screen system (3). The strategy was first used to investigate DNA-damage induced cell death in a cytokine-dependent cell line. The retroviral mutagen constructs were transfected into a retroviral packaging cell line and the viruses were harvested 48 hours post transfection (Figure 1). These viruses were then used to infect the cytokine-dependent cell line 32D cells at MOI <=1 (3). These infected cells were first cultured in flasks for 3 days in the presence of the DNA-damage inducing drug cisplatin (CDDP) at a concentration of 50μM. Under this condition, >95% of the 32D cells underwent apoptosis (Figure 2). Subsequently, the cells were washed to eliminate CDDP, and plated in 6 x 96-well plates at approximately 1,000 cells per well. Multiple clones have been isolated from this genetic screen.

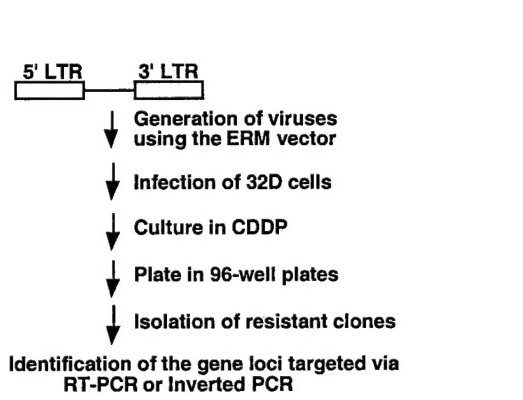

Figure 1. Flow chart for ERM-mediated genetic screen

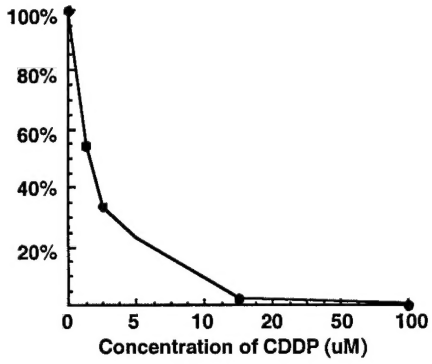

Figure 2 Survival of 32D cells in different concentrations of CDDP

The ERM vector used in the genetic screen has been engineered to contain a tetracycline responsive promoter (3). Therefore the cells that have survived can be grown in the presence or absence of tetracycline to confirm if the clones were the result of authentic retroviral integration. In this case, true retrovirally targeted cells would be more sensitive to CDDP-induced apoptosis in the presence of tetracycline. The isolated clones were thus analyzed for their responsiveness to tetracycline and only those that lost the ability to survive in the presence of both CDDP and tetracycline were analyzed further.
We then isolated RNA and genomic DNA from these individual clones and performed RT-PCR and inverted PCR respectively to identify the gene loci that were targeted by ERM. One of the gene loci targeted is the protein kinase Lyn. It hs been shown that overexpression of Lyn can protect the cells from apoptosis induced by DNA damage agents (4-7). Further studies are underway to further identify other genes that have been targeted by ERM.

For Task 2, we have proposed to biochemically characterize breast cancer genes including how BARD1 may be involved in breast cancer cell survival. BARD1 was cloned originally as an interactor of BRCA1 and has been implicated as a critical factor in BRCA1 tumor suppression (8). Missense, point mutation and loss-of-function mutations of BARD1 have been found in breast cancers (9). Our preliminary results suggest that BARD1 may modulate cell survival in response to DNA damage. To further elucidate the pathways mediated by BARD1, we took two approaches.

First, we employed the RNA interference assay (10). It has been shown that small double stranded RNAs can result in sequence specific inhibition of gene expression. Therefore, we designed and synthesized 21nt double-stranded RNA oligos with sequences homologous to that of BARD1. These siRNAs were then transfected into the human breast cancer cell line MCF-7. As shown in Figure 3, transfection of the double-stranded RNA resulted in a significant reduction in the protein expression of BARD1. We are currently in the process of assaying for how inhibition of BARD1 expression may affect cell death as a result of DNA damage. The second approach we undertook was to identify

the factors that may interact with BARD1. Experiments are underway to accomplish this. We expect more progress to be made in the near future.

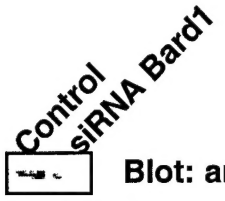

Figure 3. siRNA treatment leads to down-regulation of Bard1 protein expression.

Key Research Accomplishments

- Establishment and improvement of the ERM genetic screen approach
- Generation of CDDP resistant 32D cell clones
- Successful employment of the siRNA approach
- Establishment of a system to identify BARD1 associated factors

Reportable Outcomes

We have successfully utilized the ERM approach to identify genes that would confer resistance to CDDP induced cell death. Among the genes identified is the protein kinase Lyn. Transient BARD1 knock out cells were also generated and the approach has been confirmed to be working.

Conclusions

In summary, we have improved and successfully utilized our genetic screen approach for high efficiency mutagenesis. Multiple clones have been isolated and some of the gene loci targeted by the ERM mutagen have also been identified. Furthermore, we are well on our way to elucidate how BARD1 may interact and affect cell survival. The information obtained from our studies should prove especially useful for the development of new and effective screening strategies, drug targets, and treatment for breast cancer.

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